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Targeting the phosphoinositide 3-kinase p110- isoform impairs cell proliferation, survival, and tumor growth in small cell lung cancer

Wojtalla, Anna ; Fischer, Barbara ; Kotelevets, Nataliya ; Mauri, Francesco A ; Sobek, Jens ; Rehrauer, Hubert ; Wotzkow, Carlos ; Tschan, Mario P ; Seckl, Michael J ; Zangemeister-Wittke, Uwe ; Arcaro, Alexandre

Abstract: **PURPOSE:** The phosphoinositide 3-kinase (PI3K) pathway is fundamental for cell proliferation and survival and is frequently altered and activated in neoplasia, including carcinomas of the lung. In this study, we investigated the potential of targeting the catalytic class I(A) PI3K isoforms in small cell lung cancer (SCLC), which is the most aggressive of all lung cancer types. **EXPERIMENTAL DESIGN:** The expression of PI3K isoforms in patient specimens was analyzed. The effects on SCLC cell survival and downstream signaling were determined following PI3K isoform inhibition by selective inhibitors or downregulation by siRNA. **RESULTS:** Overexpression of the PI3K isoforms p110- and p110- and the antiapoptotic protein Bcl-2 was shown by immunohistochemistry in primary SCLC tissue samples. Targeting the PI3K p110- with RNA interference or selective pharmacologic inhibitors resulted in strongly affected cell proliferation of SCLC cells in vitro and in vivo, whereas targeting p110- was less effective. Inhibition of p110- also resulted in increased apoptosis and autophagy, which was accompanied by decreased phosphorylation of Akt and components of the mTOR pathway, such as the ribosomal S6 protein, and the eukaryotic translation initiation factor 4E-binding protein 1. A DNA microarray analysis revealed that p110- inhibition profoundly affected the balance of pro- and antiapoptotic Bcl-2 family proteins. Finally, p110- inhibition led to impaired SCLC tumor formation and vascularization in vivo. **CONCLUSION:** Together our data show the key involvement of the PI3K isoform p110- in the regulation of multiple tumor-promoting processes in SCLC.

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Targeting the Phosphoinositide 3-Kinase p110 α Isoform Impairs Cell Proliferation, Survival and Tumor Growth in Small Cell Lung Cancer

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Running title: Targeting PI3K signaling in SCLC

Keywords: apoptosis; Bcl-2; PI3K; proliferation; small cell lung cancer

Conflicts of interest: The authors declare that there is no conflict of interest.

Abstract

Purpose: The phosphoinositide 3-kinase (PI3K) pathway is fundamental for cell proliferation and survival and is frequently altered and activated in neoplasia, including carcinomas of the lung. In this study, we investigated the potential of targeting the catalytic class I_A PI3K isoforms in small cell lung cancer (SCLC), which is the most aggressive of all lung cancer types.

Experimental Design: The expression of PI3K isoforms in patient specimens was analyzed. The effects on SCLC cell survival and downstream signaling were determined following PI3K isoform inhibition by selective inhibitors or down-regulation by small interfering RNA.

Results: Over-expression of the PI3K isoforms p110 α and p110 β and the anti-apoptotic protein Bcl-2 was shown by immunohistochemistry in primary SCLC tissue samples. Targeting the PI3K p110 α with RNA interference (RNAi) or selective pharmacological inhibitors resulted in strongly affected cell proliferation of SCLC cells *in vitro* and *in vivo*, while targeting p110 β was less effective. Inhibition of p110 α also resulted in increased apoptosis and autophagy, which was accompanied by decreased phosphorylation of Akt and components of the mammalian target of rapamycin (mTOR) pathway, such as the ribosomal S6 protein, and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). A DNA microarray analysis revealed that p110 α inhibition profoundly affected the balance of pro- and anti-apoptotic Bcl-2 family proteins. Finally, p110 α inhibition led to impaired SCLC tumor formation and vascularization *in vivo*.

Conclusion: Together our data demonstrate the key involvement of the PI3K isoform p110 α in the regulation of multiple tumor-promoting processes in SCLC.

Translational relevance

Small cell lung cancer (SCLC) is a common form of lung cancer and is associated with very unsatisfactory survival rates. Novel therapies are thus urgently required and will arise from a better understanding of the disease biology. We were interested in gaining further insight into the potential of targeting PI3K isoforms in SCLC. In this report, we show that the catalytic p110 α isoform is over-expressed in a subset of primary SCLC samples. The growth of SCLC cells was impaired on targeting p110 α using RNA interference or specific pharmacologic inhibitors. Inhibition of p110 α also induced apoptosis and autophagy, which was paralleled by a decrease in the expression levels of anti-apoptotic Bcl-2 family proteins. Importantly, SCLC tumors treated with p110 α inhibitors displayed reduced proliferation and enhanced apoptosis *in vivo*. Together, the results presented in this study show that specific p110 α inhibitors may in the future represent new drugs for SCLC.

Introduction

Lung cancer is a major cause of death in the developed world and the commonest cancer killer in men. Small cell lung cancer (SCLC) represents about 13-15% of all cases of lung cancer and is strongly associated with cigarette smoking. Combinatorial chemotherapy regimens with etoposide and platinum-based agents, as well as radiotherapy, are commonly used for the treatment of SCLC patients. However, an initial therapeutic response is usually followed by disease recurrence within less than 1 year and therefore the overall 5-year survival rate is <5%. Consequently, novel therapeutic strategies are urgently required for SCLC. In the past years, an increasing number of molecular alterations involved in SCLC pathogenesis have been reported, including ectopic expression of neuroendocrine regulatory peptides, up-regulation of anti-apoptotic Bcl-2 proteins, overexpression of *myc* family oncogenes and extracellular matrix proteins, as well as genetic abnormalities in the tumor suppressor genes *TP53* and *RB* (1, 2). In addition, it has been shown that polypeptide growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1) and stem cell factor (SCF) control key biological responses in human SCLC cells, including growth and proliferation, chemoresistance, and migration (3-6). Downstream of activated polypeptide growth factor receptors, activation of two major intracellular signaling cascades, the phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and the mitogen-activated Erk kinase (MEK)/extracellular signal-regulated kinase (Erk) pathway, have been found to be involved in the survival and proliferation of SCLC (3, 5-8). Furthermore, it has been reported that mTOR and the ribosomal protein S6 kinases (S6K) are over-expressed in SCLC cells, compared with normal human type II pneumocytes (6). Therefore, targeting these pathways with novel selective pharmacological inhibitors may lead to the development of more effective therapies for SCLC.

The PI3K signaling pathway controls key cellular responses, such as cell growth and

proliferation, survival, migration and metabolism. Over the last decades, it has been recognized that this intracellular signaling pathway is frequently activated by genetic and epigenetic alterations in human cancer, including lung cancer. The PI3K family of signaling enzymes comprises eight catalytic isoforms, which are subdivided into three classes. The class I_A PI3K isoform p110 α is considered to be a validated drug target in human cancer (9-11), in particular because activating mutations in the *PIK3CA* frequently occur in human cancer. In SCLC, amplification and mutations of the *PIK3CA* gene were identified and the p110 α and p110 β isoforms were found over-expressed in cell lines, in addition to deregulation of the PI3K/Akt/mTOR pathway (12).

Here we report for the first time that targeting the class I_A PI3K isoform p110 α blocks SCLC cell growth and survival *in vitro* and *in vivo* and present evidence that this isoform plays a crucial role in Akt/mTOR pathway activation and Bcl-2 family protein expression.

Materials and methods

Antibodies and reagents

Antibodies: Caspase-3, poly-(ADP-ribose)-polymerase (PARP), Akt, PI3K p110 β (Santa Cruz Biotechnology), p-Akt^{Ser473}, p-Bcl2^{Ser70}, Bcl2, Bcl-X_L, Bax, Bad, p-4EBP1^{Thr37/46}, p-S6^{Ser235/236} or p-S6^{Ser240/244}, S6 protein, PI3K p110 α (Cell Signaling Technology), β -actin (Sigma Aldrich), Mcl-1, NF- κ B (Epitomics). Etoposide, chloroquine, z-VAD-FMK, IKK inhibitor/wedelolactone (Calbiochem). RAD001 was supplied by the Novartis Institutes for BioMedical Research Basel, Oncology, Switzerland. The PI3K inhibitors PIK75, YM024, TGX221 and PI103 were the kind gift of Prof. Shaun Jackson.

Cell lines, cell culture, and cell proliferation

SCLC cell lines were obtained from the American Type Culture Collection (Suppl. Table 2). The human SCLC cell lines H69, H209, H510, and SW2 were cultured in RPMI medium containing 10% heat-inactivated FCS. Cell viability was analyzed by using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega).

Reverse transcription-PCR and TaqMan analysis

Total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instruction. For each reverse transcription-PCR, 1 μ g of total RNA was used with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction conditions were used according to the manufacturer's protocol. TaqMan Gene Expression Assays for the PI3Ks p110 α and p110 β , as well as for VEGFA were done according to the manufacturer's instructions (Applied Biosystems). As internal control the expression of GAPDH

was analyzed in each sample, separately.

Immunohistochemistry

Immunoreactivity was evaluated on commercial TMA sections of SCLC (Biomax LC10010 (2 cores; Female 9 (22.5%), Female age 32-66 yo (mean value 52.5); Male 31 (77.5%), Male age 34-76 yo (mean value 53.0); Stage I 11, Stage II 20, Stage IIIa 7, Stage IIIb 2; Node negative 12, Node positive 28 (22 N1 and 6 N2)) using the PI3K p110 α (Cell Signaling Technology, 4249), p110 β (Abcam, ab55593) and Bcl-2 (Cell Signaling) antibodies in a modification of the antigen retrieval technique (13). The antibodies were used at a 1:200 dilution for 1 h at room temperature and then processed with Polymer-HRP Kit (BioGenex, San Ramon CA) with diaminobenzidine development and Mayer Haematoxylin counterstaining. Breast cancer tissue was used as positive external control. Negative controls were obtained by omitting the primary antibody. A semi quantitative immunohistochemical score (IHS) was used, including assessment of both the percentage of positive cells and the intensity of staining. For the intensity, a score of 0 to 3, corresponding to negative, weak, moderate and strong positivity, was recorded. The range of possible scores was thus 0 to 300. IHS and similar semi-quantitative scoring systems have been successfully used for TMA evaluation. Each core was scored individually. One observer scored all cases, which were rechecked randomly by the same investigator after a period of time.

Serial sections of paraffin-embedded CAM tumors were de-waxed in xylol and rehydrated through graded dilutions of ethanol. Antigen recovery was performed with citrate buffer (10mM) in a microwave (800 watts) for 7 min. Endogenous peroxidase activity was blocked with 3% H₂O₂. Sections were incubated in a humid chamber at 4 °C overnight with or without (negative controls) active caspase-3 (Abcam, ab2302; recognizes the cleaved active p17 fragment) and Ki67 (Novus Biologicals, NB110-57147) antibodies. Biotin-conjugated goat anti-Rabbit (Dako, E0432) and avidin-biotin-horseradish peroxidase complex (Vector Laboratories,

Vectastain ABC Kit, PK6100) were used. Bound peroxidase was detected with 3, 3'-diaminobenzidine substrate. Sections were subsequently counterstained with Hematoxylin and mounted in Aquatex (Merck).

Transient transfection and stable transduction of SCLC cells

SCLC cells were transiently transfected with either non-targeting small interfering RNA (siRNA) or SMARTpool siRNA duplexes targeting the PI3K p110 α or PI3K p110 β , using the Amaxa Nucleofector system (Amaxa Biosystems), according to the manufacturer's protocol. Briefly, 1.5×10^6 SCLC cells grown in RPMI/10% FCS were transfected with 6 μ L of 20 μ mol/L siRNA in 100 μ L of Nucleofector Solution V using the program T-16 on the Amaxa Nucleofector. Following transfection, cells were transferred into RPMI/10 % FCS overnight, before they were used for experiments.

Lentiviral vectors expressing small hairpin (sh)RNA constructs specifically targeting the PI3K p110 α (Sigma-Aldrich) were tested for stable knockdown efficiency. The constructs shPI3KC_2892 and shPI3KC_3433 were chosen for further experiments since they most efficiently silenced p110 α . The non-targeting vector SHC002 was used as a control. Lentivirus production and transduction of cells was done as described before (14). The vectors contain a puromycin resistance gene and stably transduced cell populations were selected for two weeks using puromycin at the concentration 1.5 μ g/ml.

In vivo experiments

The effect of the PI3K inhibitor PIK75 on the growth and SCLC tumor formation *in vivo* were assessed on the chorioallantoic membrane (CAM) of chick embryos (15). Briefly, 3×10^6 H69 SCLC cells were placed in RPMI containing 50% BD Matrigel Matrix (BD Biosciences) on the CAM on embryonic day 9. Increasing concentrations of the p110 α inhibitors PIK75 were deposited with the SCLC cells. Controls were treated with the solvent of the corresponding drug.

CAMs were examined for vessel formation under a stereomicroscope. Tumor size and density of vessels per area around the tumor were determined using the software Vessel_tracer(16). The application of Matrigel without/with PIK75 on the CAM was used as a negative control (Suppl. Fig. 3).

SDS-PAGE and Western blotting

The assays were done as described before (3).

DNA microarray

The cDNA microarray analysis was performed at the Functional Genomic Center of the University of Zurich. Gene expression data were obtained by hybridizing Human Genome U133 Plus 2.0 Affymetrix Gene Chips arrays, on which >54000 transcripts were represented. Each experiment represented a group of three independent biological replicates. Raw data generated by the GCOS Software (Affymetrix) were processed by using the RMA method (17) and further statistically analyzed by using the software R and applying Student's t-test. The GeneGO MetaCore (GeneGO, St Joseph, MI, USA) was used to define functional annotations for the selected genes, thus assigning them to ontological categories for association with relevant biological processes and pathways. The accession number of the data in Gene Expression Omnibus is GSE40564.

Results

Expression of the PI3K p110 α and p110 β and the anti-apoptotic protein Bcl-2 in SCLC patient samples

Our previous work in SCLC cell lines had revealed that class I_A PI3K isoforms are over-expressed in comparison to type II pneumocytes, which are one of the precursors of SCLC. To investigate whether this finding could be confirmed in primary tumors, a SCLC tissue microarray was stained with antibodies specific for p110 α or p110 β (Fig. 1A). This analysis revealed that, while normal lung tissue did not express detectable levels of either PI3K isoforms, p110 α and p110 β displayed enhanced expression in subsets of primary SCLC (Fig. 1A-B). Around 25% of SCLC tumors showed over-expression of p110 α , while 18% of cases were positive for p110 β (Fig. 1B). In general, SCLC tumors did not display concomitant over-expression of p110 α and p110 β , which was only present in 5% of the tumors (data not shown). Additionally, the expression of the anti-apoptotic protein Bcl-2 was assessed in SCLC patient samples. The IHC staining of a TMA with a specific antibody revealed a high Bcl-2 over-expression in ~90% (35/39 cases) of the SCLC patient samples (Fig. 1C+D), compared to normal lung tissue, which did not display detectable levels of Bcl-2.

Class I_A PI3K p110 α inhibition or silencing blocks cell proliferation and Akt/mTOR signaling

Previous work had shown that targeting the PI3K/Akt/mTOR pathway by using small molecule inhibitors impaired SCLC proliferation *in vitro* and *in vivo*. Therefore, we investigated the impact of isoform-specific inhibitors of class I_A PI3K isoforms on the proliferation of SCLC cell lines. The potent p110 α inhibitor PIK75 (IC₅₀ *in vitro* = 7.8 \pm 1.7 nM) (18) significantly impaired the proliferation of 4 SCLC cell lines with IC₅₀ values in the range of 50-100 nM (Fig. 2A). The p110 α inhibitor YM024, which is much less potent against the enzyme *in vitro* (IC₅₀ *in vitro* = 0.3 μ M),

also impaired the proliferation of the SCLC cell line panel, but at higher concentrations (Fig. 2A). The potent p110 β inhibitor TGX-221 (IC₅₀ *in vitro* = 8.5 \pm 0.9 nM) (18) only partially impaired the proliferation of SCLC cell lines, but at high concentrations (Fig. 2B). In contrast, the p110 δ inhibitor IC87114 did not significantly impair the proliferation of the cell lines under study (Fig. 2B). We also evaluated the impact of the dual p110 α /mTOR inhibitor PI103 in the panel of SCLC cell lines. PI103 displayed anti-proliferative activity against the SCLC panel *in vitro*, with IC₅₀ values in the range of 100-500 nM (Suppl. Fig. 1A).

The impact of the selective PI3K inhibitors on the activation status of the Akt/mTOR pathway in SCLC cell lines was then investigated by Western blot analysis (Fig. 2C). PIK75 and PI103 strongly affected the activation status of Akt and the phosphorylation of the mTOR downstream targets ribosomal S6 protein and 4E-BP1 (Fig. 2C; Suppl. Fig. 1B). YM024 also impaired Akt activation and partially inhibited mTOR pathway activation (Fig. 2C; Suppl. Fig. 1B). In contrast, TGX221 was less effective at blocking the activation of the Akt/mTOR pathway (Fig. 2C; Suppl. Fig. 1B).

To validate our findings with pharmacological inhibitors, we used RNA interference to down-regulate the expression of p110 α or p110 β in SCLC cell lines. Transient down-regulation of p110 α strongly impaired the activation of Akt and phosphorylation of the ribosomal S6 protein, while p110 β silencing was ineffective (Fig. 3A). In addition, p110 α silencing induced a significant decrease in the proliferation of SCLC cells (40% reduction), while p110 β down-regulation was less effective (25% reduction) (Fig. 3B). We also used lentiviral delivery of short hairpin RNAs targeting p110 α in SCLC cell lines. Also this approach resulted in stable silencing of p110 α , robustly affected PI3K downstream signaling (Fig. 3C) and SCLC cell proliferation was partially impaired by shRNA targeting of p110 α (Fig. 3D).

Together these results show that targeting p110 α selectively impairs cell proliferation and activation of the Akt/mTOR pathway in SCLC cell lines *in vitro*.

Class I_A PI3K p110 α inhibition induces apoptosis and autophagy in SCLC cell lines

Previous work had shown that targeting the PI3K/Akt/mTOR pathway by using small molecule inhibitors (7, 19) induced apoptosis in SCLC cell lines. Therefore, we investigated the impact of isoform-specific inhibitors of class I_A PI3K isoforms on the survival of SCLC cell lines. Treatment of a panel of SCLC cell lines with PIK75 or YM024 induced cleavage of PARP and reduction of pro-caspase3, which are markers of apoptosis induction (Fig. 4A), whereas TGX221 was less efficient at inducing apoptosis (Fig. 4A). The induction of apoptosis upon p110 α inhibition appeared to be stronger in the SCLC cell lines H69 and H209, compared to the effects observed in H510 (intermediate) and SW2 (low) (Fig. 4A). The caspase inhibitor zVAD-FMK was able to rescue 20% of the PIK75-induced apoptosis in H69 cells and 10% of the TGX221-induced apoptosis ($p < 0.01$) (Fig. 4B). Because the strongly affected cell viability in SCLC cells upon p110 α inhibition could not only be explained by the apoptotic cell response, we further investigated other cell death mechanisms, such as autophagy. Whereas the inhibition of p110 α with PIK75 or YM024 induced an increased conversion of LC3-I to LC3-II, which is indicative for autophagic activity, a comparable response could not be observed in cells where p110 β was inhibited (Fig. 4C, Suppl. Fig. 2). ATG5, another protein used as a typical marker for autophagy, was also expressed in SCLC cells. Compared to the vehicle-treated control, enhanced ATG5 expression was not, or only slightly, observed in PIK75-treated H69 cells (Fig. 4C). Interestingly, at later time points, a decrease in ATG5 expression was observed (data not shown). Chloroquine is known to inhibit autophagic activity due to de-acidification of lysosomes, followed by accumulation of ineffective autophagic vesicles. In H69 cells treated with the PI3K inhibitors PIK75 and TGX221, chloroquine was able to rescue 20% (and 10% respectively) of the autophagy-induced decrease in cell viability (Fig. 4D), suggesting additive roles, or crosstalk, between autophagy and apoptosis in p110 α -induced cell death.

Inhibition of the PI3K p110 α impairs SCLC tumor formation and vascularization in vivo

Together the data collected from our experiments and the known literature indicated an important role for p110 α in SCLC cell growth and survival processes *in vitro* and *in vivo*. To further investigate its impact on tumor formation and maintenance, an *in vivo* assay was employed to grow vehicle- and PIK75-treated H69 cells on the chorioallantoic membrane (CAM) of chick embryos. Tumor formation was clearly impaired upon PIK75 treatment, which was shown by strongly reduced tumor size and tumor weight compared to the control treatment (Fig. 5A). Additionally, IHC staining of PIK75-treated tumor sections showed a decreasing expression of the proliferation marker Ki67 and a rising expression of the apoptotic marker cleaved caspase-3 with increasing PIK75 concentrations (Fig. 5C). Finally, p110 α inhibition was able to partially prevent the vascularization on the CAM around the tumorigenic area, as measured by the vessel density (Fig. 5B). Another indication of impaired tumor vascularization upon p110 α inhibition is the reduced VEGFA expression observed in the DNA microarray and quantitative RT-PCR analysis of PIK75-treated H69 cells (data not shown).

DNA microarray analysis of SCLC cells treated with isoform-specific PI3K inhibitors

In order to investigate whether the class I_A PI3K isoform p110 α controls the expression of specific gene subsets in SCLC, we performed DNA microarray analysis in H69 cells treated with either vehicle, PIK75 targeting p110 α , or TGX221 targeting p110 β (Fig. 6A). The efficacy of the down-regulation of the Akt/mTOR pathway by the respective inhibitors was demonstrated by Western blot analysis, as well as quantitative RT-PCR for VEGFA expression (Fig. 2C and data not shown). Inhibiting p110 α significantly affected the expression of 3411 genes (P-value 0.01; FC \geq 1.5), while inhibiting p110 β resulted in significant changes (P-value 0.01; FC \geq 1.5) in 4 genes, suggesting an important role of p110 α , but not p110 β , for the regulation of the expression of a subset of genes in SCLC (Fig. 6A). We next sought to investigate which genes were selectively affected by p110 α inhibition, in comparison to p110 β . Amongst these genes, anti-

apoptotic proteins of the Bcl-2 family of proteins were found to be more significantly down-regulated in SCLC cells treated with p110 α inhibitor, than in the case of the p110 β inhibitor (Table 1).

The proteins of the Bcl-2 family are downstream targets of p110 α

In order to validate the Bcl-2 family proteins as targets of p110 α in SCLC cell lines, we used antibody arrays and Western blot analysis to confirm the results obtained by DNA microarrays. An analysis using antibody arrays revealed impaired Akt pathway activation and down-regulation of anti-apoptotic Bcl-2 family proteins (data not shown). Down-regulation of the expression and impaired activation of the anti-apoptotic Bcl-2 family proteins could indeed be demonstrated at the protein level upon p110 α inhibition (Fig. 6B). As was already observed previously for the apoptotic response, the SCLC cell lines under study responded varyingly to p110 α inhibition. In particular, the cell lines H69 and H209 displayed a strongly decreased expression of the Bcl-2 family members Bcl-2, Bcl-X_L and Mcl-1, whereas SW2 cells appeared to be more resistant. In addition, p110 α inhibition induced the levels of the pro-apoptotic family members Bad and Bax (Fig. 6B). In contrast to the inhibitors of p110 α , TGX221 had little effect on the expression levels of Bcl-2 family proteins (Fig. 6B). The anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-X_L are known targets of the NF- κ B transcriptional network, which controls different cell responses including immune response, cell proliferation and survival (20). To gain insight into the transcriptional networks affected by silencing of p110 α in SCLC cells, we performed a biostatistical analysis of the gene expression data, using GeneGo. The transcriptional networks that were most significantly altered comprised HNF4 α , SP1 and c-Myc, the estrogen receptor (ER) and also NF- κ B (Suppl. Table 1). We could indeed observe a deregulation of NF- κ B expression in H69 cells treated with PIK75 and YM024, whereas p110 β inhibition did not have any effect (Fig. 6C). These results suggested NF- κ B to be a downstream target of PI3K p110 α signaling in SCLC and, therefore, we assumed that targeting NF- κ B may have an effect on the

expression of Bcl-2 and Bcl-X_L. This hypothesis was confirmed by the observation that the expression levels of Bcl-2 were indeed decreased in H69 cells treated with wedelolactone, an inhibitor of the I κ B-kinase (IKK) (Fig. 6D).

Discussion

The PI3K/Akt/mTOR pathway has been demonstrated to play a key role in SCLC cell proliferation, survival, chemoresistance and migration. Mutations in *PIK3CA* and gene amplification were reported in primary SCLC, as well as increased expression of *PIK3CA* at the mRNA and protein level. Broad specificity PI3K/mTOR inhibitors have shown anti-tumor activity in SCLC models *in vitro* and *in vivo* (21). In this report, we have investigated the impact of isoform-specific inhibitors and RNAi targeting class I_A PI3K isoforms on SCLC cell responses. In general, agents targeting p110 α reduced SCLC cell proliferation *in vitro*, impaired the SCLC tumor formation, which was accompanied by affected vascularization *in vivo*, and decreased the activation status of classical PI3K downstream targets, such as Akt, mTOR and S6K. Targeting p110 α by RNAi or isoform-specific inhibitors had more pronounced effects on SCLC cell responses than in the case of p110 β or p110 δ , indicating a selective role for p110 α in SCLC. In view of these observations, we hypothesized that p110 α may control the expression of a selective subset of genes implicated in SCLC cell proliferation and/or survival. The comparative DNA microarray analysis of SCLC cell lines in which either p110 α or p110 β were inhibited by selective compounds identified such a gene subset. The Bcl-2 family of proteins was validated as a downstream target of p110 α by a combination of approaches. The observation that Bcl-2 expression was elevated in primary SCLC, in comparison to normal lung tissue further supports this model, in view of the over-expression of p110 α . Importantly, the Bcl-2 family of proteins has been previously shown to play a crucial role in the survival of SCLC cell lines *in vitro* and *in vivo* (5, 22-26). The p110 α inhibitors induced increases in both SCLC apoptosis and autophagy, which is consistent with Bcl-2 family proteins being a target of p110 α . Bcl-2 family proteins are key regulators of both apoptosis and autophagy (27) and their reduced expression upon inhibition of the p110 α /NF κ B pathway may play an essential role in the effects of the

p110 α inhibitors in SCLC.

We have previously evaluated the mTOR inhibitor everolimus in SCLC cell lines and found that it was effective in a subset of SCLC cell lines characterized by activation of the Akt/mTOR pathway and low expression levels of anti-apoptotic Bcl-2 family proteins (24). In view of the results obtained with isoform-selective inhibitors of p110 α , it can be speculated that these agents may be more potent, since they induce a down-regulation of anti-apoptotic Bcl-2 family proteins and of the activity of the Akt/mTOR pathway. This may be particularly relevant for SCLC tumors bearing activating mutations in the *PIK3CA* gene.

Interestingly, our previous work in neuroblastoma has shown that the class I_A PI3K isoform p110 δ contributes to cell proliferation and survival by controlling the activation of the mTOR pathway and the expression levels of anti-apoptotic Bcl-2 family proteins. Overexpression of p110 δ was found in a subset of primary neuroblastoma and cell lines, and p110 δ was essential for signal transduction by receptor tyrosine kinases, such as the IGF-1R and EGFR (28). In contrast, in SCLC cell lines, p110 α appears to play a more important role in the activation of the Akt/mTOR pathway, which may explain the impact of agents targeting this isoform on SCLC proliferation and survival. A previous report has shown that the activity of any class I_A PI3K isoform can maintain cell survival (29). Therefore, the relative importance of class I_A PI3K isoforms in selected cancer types may be, in part, attributed to differences in expression levels.

Thus, targeting PI3K p110 α signaling may represent an attractive novel approach to develop novel therapies for SCLC. Indeed, different pharmacological inhibitors of this isoform now exist, which will soon enter clinical trials in oncology, and could, in the future, be developed as new drugs for SCLC.

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Figure legends

Fig. 1 Expression of the PI3K p110 α and p110 β , and the anti-apoptotic protein Bcl-2 in SCLC patient samples. (a) Immunostaining with PI3K p110 α and p110 β showing negative SCLC staining and representative p110 α and p110 β SCLC positive staining, compared to normal lung tissue. (b) Analysis of the IHC staining PI3K p110 α and p110 β in SCLC patient samples. (c) Immunostaining with Bcl-2 showing negative and representative Bcl-2 intermediate and strongly positive SCLC staining. (b) The mean immunohistochemical staining scores (IHC scores) Bcl-2 in 20 paraffin-embedded specimens each of normal lung tissue and of 40 SCLC paraffin-embedded specimens. IHC scores = percentage of positive cells x staining intensity (for details see "Material and Methods").

Fig. 2 PI3K inhibition blocks cell viability and PI3K downstream signaling. (a+b) The SCLC cell lines H69, H209, H510, and SW2 were incubated with increasing concentrations of the class I PI3K inhibitors PIK75, YM024 (a), TGX221, and IC87114 (b) in serum-containing medium. Cell viability was assessed using the MTS assay after 3 days. The data are mean with SD from four replicates and at least three independent experiments. (c) H69 cells were incubated with increasing concentrations of the PI3K p110 α inhibitors PIK75, YM024, and PI103 (PI3K p110 α /mTOR inhibitor) and the PI3K p110 β inhibitor TGX221. After 24 h, the cells were harvested and whole cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated.

Fig. 3 Silencing of p110 α but not p110 β affects cell viability and PI3K downstream signaling activation. (a+c) H69 cells were transiently transfected with siRNA constructs targeting p110 α or p110 β , or non-targeting scrambled control (a) or p110 α was stably silenced by the lentiviral delivery of shRNA constructs (c). Cell lysates were analyzed by SDS-PAGE and

Western blotting with antibodies for the proteins indicated. (b+d) Cell viability of H69 cells transiently (b) or stably (d) transfected with constructs targeting p110 α or p110 β was assessed using the MTS assay after 2 and 3 days. A non-targeting construct was used as control.

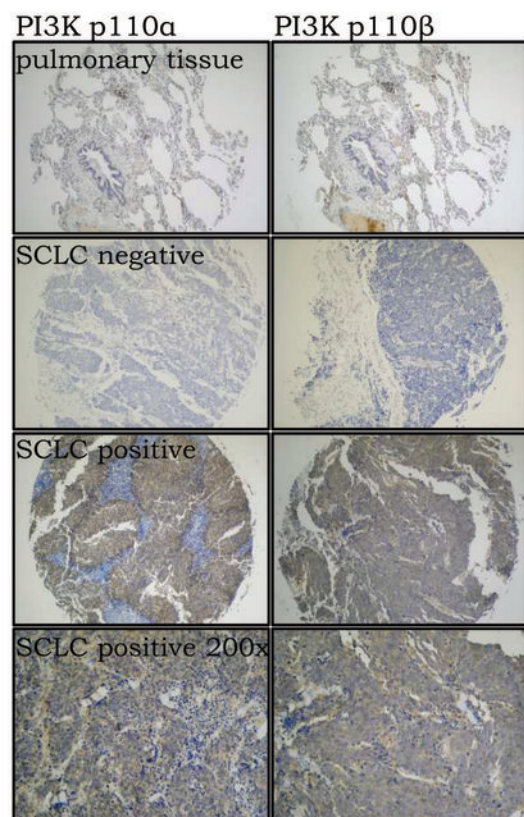
Fig. 4 PI3K inhibition increases apoptosis and autophagy in SCLC. (a+c) H69, H209, H510 and SW2 cells grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110 α inhibitors PIK75 and YM024, the PI3K p110 β inhibitor TGX221, PI103 (PI3K p110 α /mTOR inhibitor), RAD001 or etoposide/cisplatin. After 24 hours the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated. (b+d) H69 cells grown in serum-containing medium were incubated with the PI3K inhibitors PIK75 (0.05 μ M) and TGX221 (10 μ M), rapamycin (0.1 μ g/ml) or etoposide (10 μ M) in absence or presence of zVAD-FMK (50 μ M) or chloroquine (20 μ M). Cell proliferation was assessed using the MTS assay after 12h. The data are mean of four replicates and three independently performed experiments. ** $p < 0.01$, * $p < 0.05$.

Fig. 5 PI3K inhibition reduces tumor formation and vascularization *in vivo*. (a) H69 cells were treated with vehicle or different concentrations of PIK75 and applied on the CAM of chick embryos. On day 13, pictures and tumors were taken to analyze tumor size, weight (a) and vessel density (b). (b) Analysis of the vessel density reduction upon PI3K p110 α inhibition in vehicle- or PIK75-treated H69 tumors (c) Immunostaining with Ki67 and cleaved caspase-3 comparing the expression in vehicle- and PIK75-treated SCLC tumor sections. (d) Analysis of Ki67 or caspase-3 positively stained tumor cells comparing vehicle- and PIK75-treated H69 tumor sections. Positively stained cells [%] = number of positive cells/total number of cells x 100. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 6 The proteins of the Bcl-2 family are downstream targets of p110 α . (a) Gene expression analysis by DNA microarray: Heatmap of gene expression changes caused by p110 α inhibition. (b+c+d) H69, H209 and SW2 (b) or H69 (c+d) cells grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110 α inhibitors PIK75 and YM024, the PI3K p110 β inhibitor TGX221, RAD001 and etoposide/cisplatin or the IKK inhibitor wedelolactone (d). After 24 h, the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated.

Figure 1

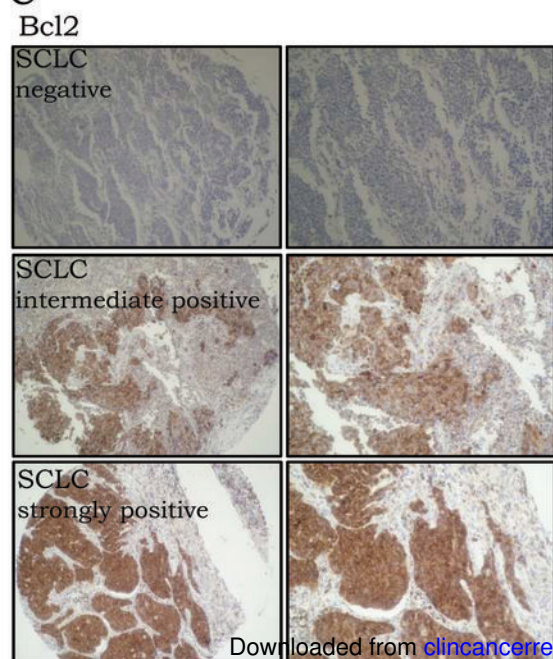
A



B

	<i>number of specimens</i>	<i>positive</i>	<i>%</i>
p110α			
<i>normal lung tissue</i>	20	0	0
<i>SCLC patient samples</i>	80	20	25
<i>T1</i>	26	3	12
<i>T2</i>	46	15	33
<i>T3</i>	4	1	25
<i>T4</i>	4	3	75
p110β			
<i>normal lung tissue</i>	20	0	0
<i>SCLC patient samples</i>	80	14	18
<i>T1</i>	26	4	15
<i>T2</i>	46	10	22
<i>T3</i>	4	1	25
<i>T4</i>	4	0	0

C



D

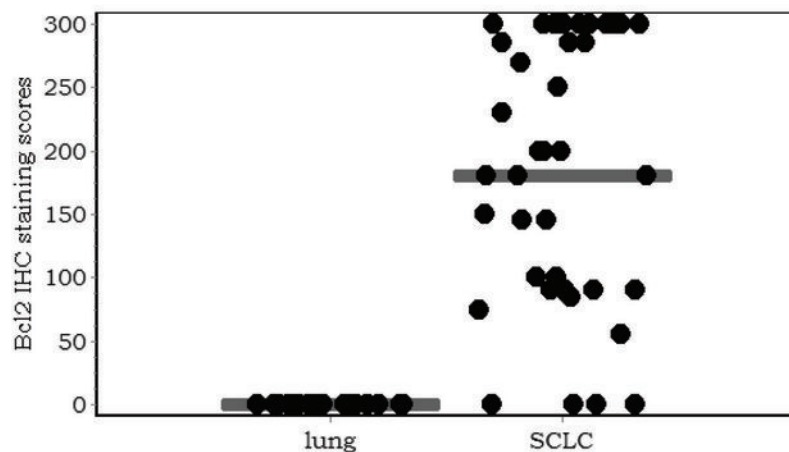


Figure 2

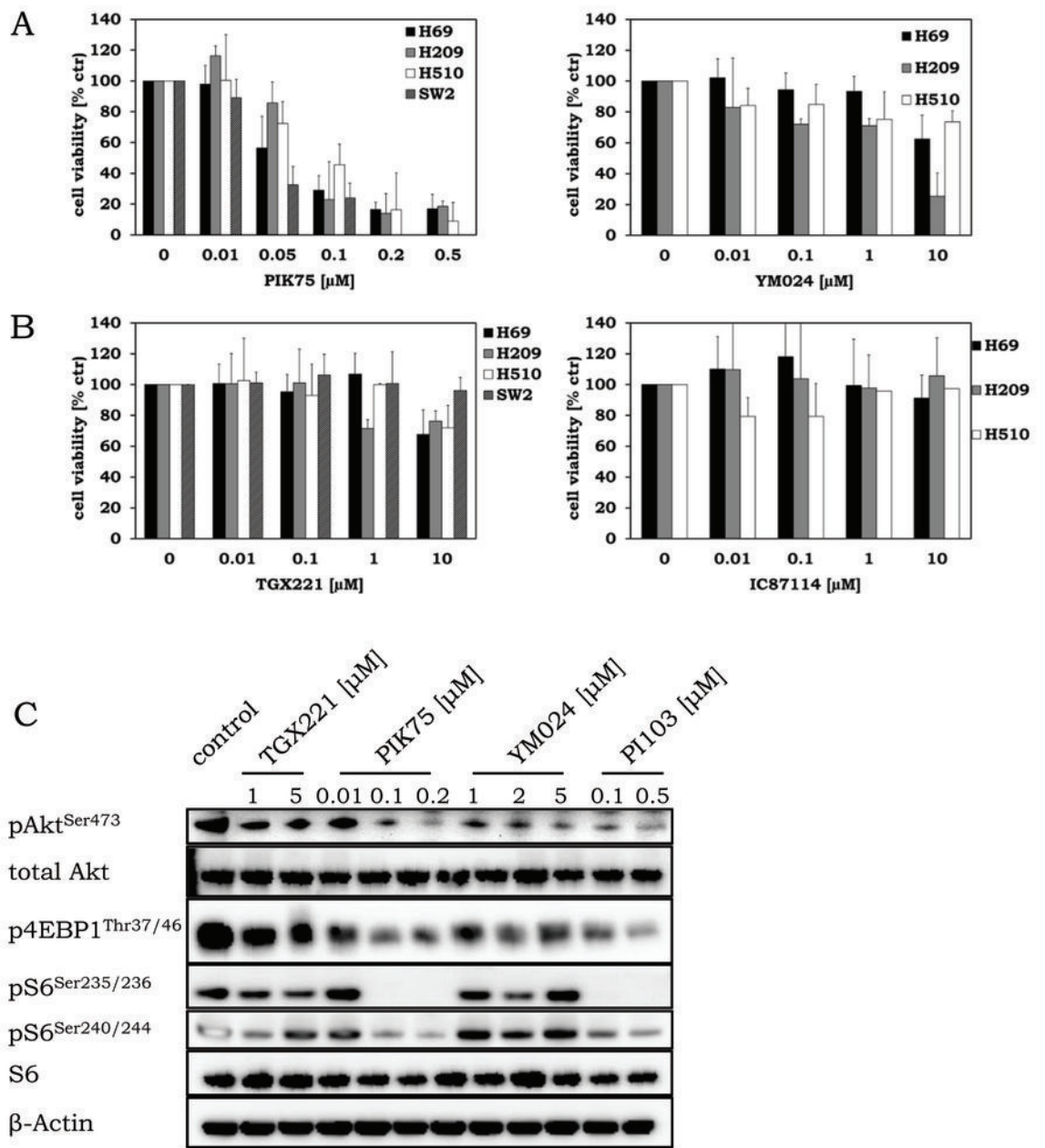
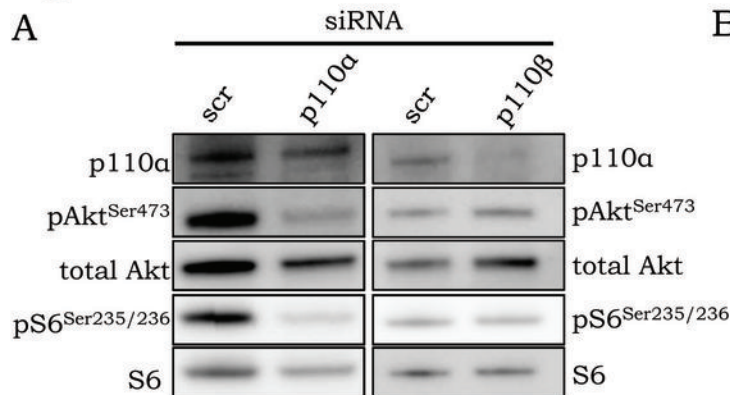
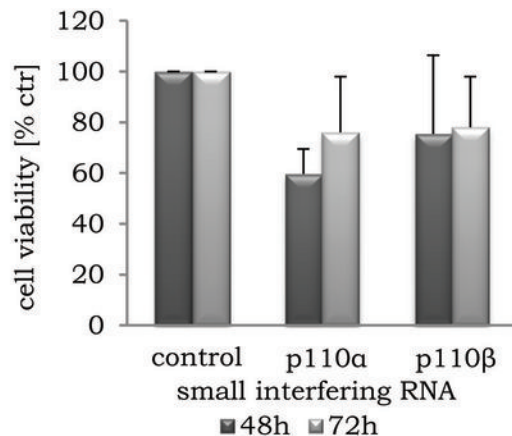


Figure 3

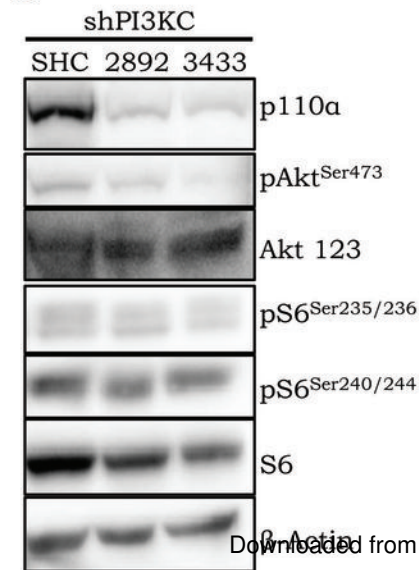
A



B



C



D

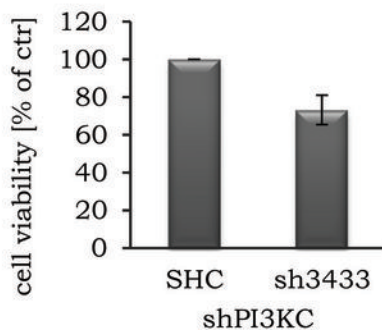


Figure 4

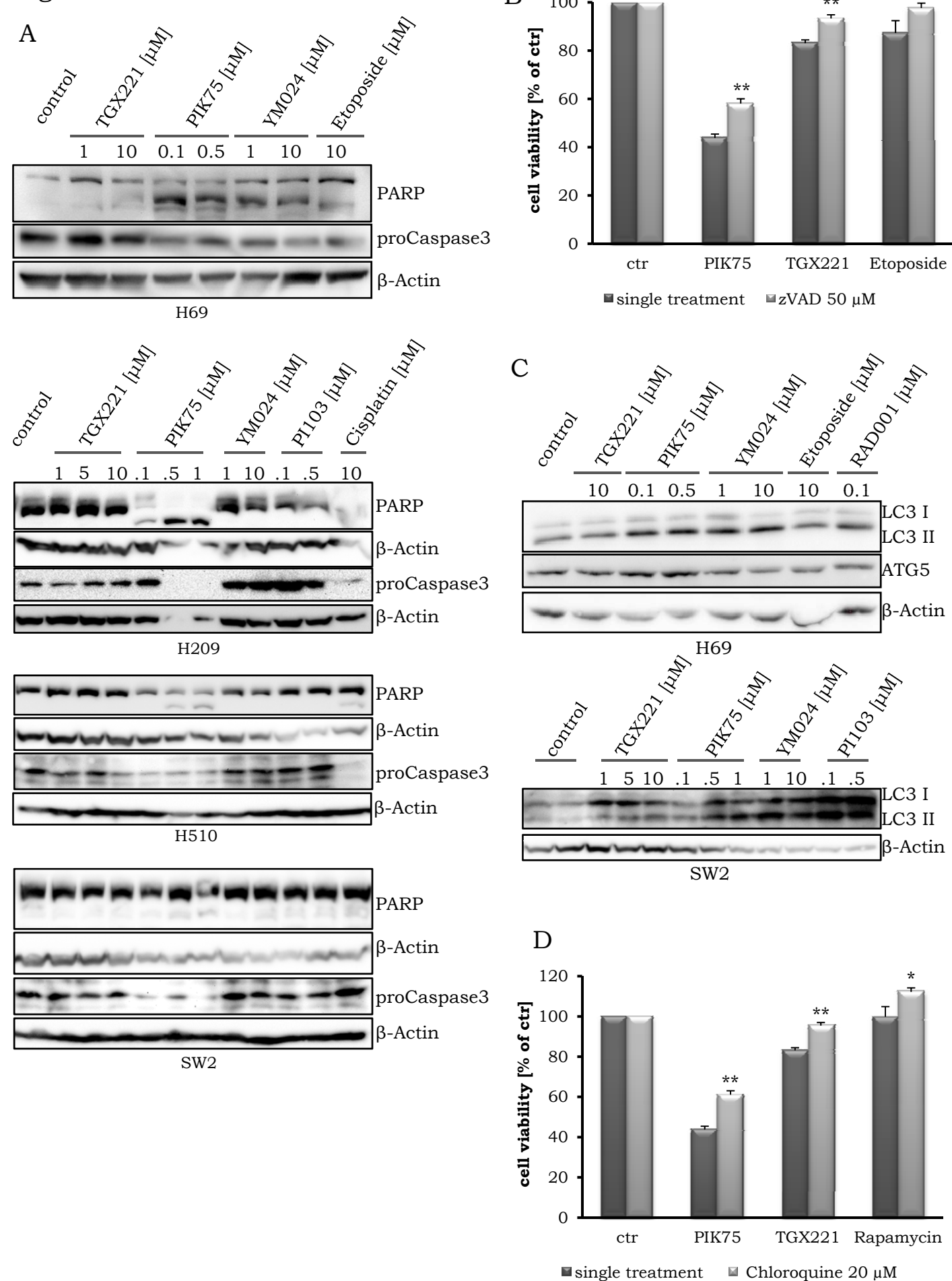
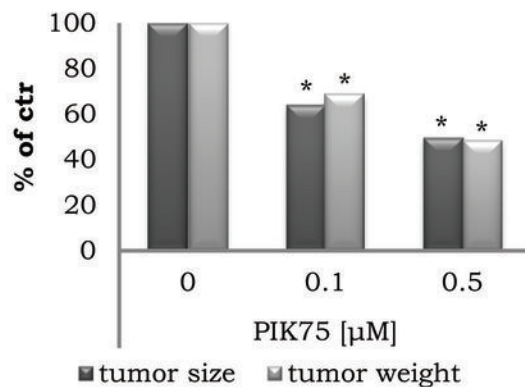
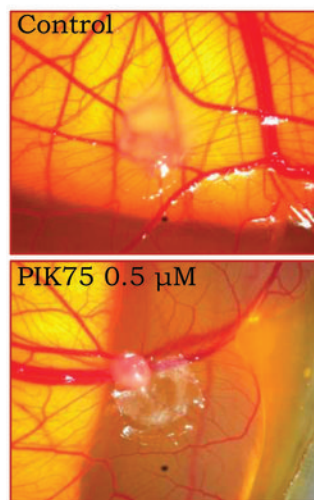
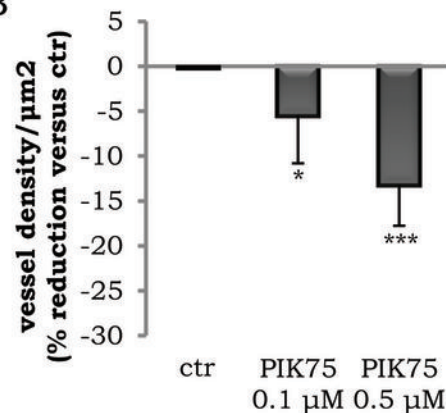


Figure 5

A



B



C

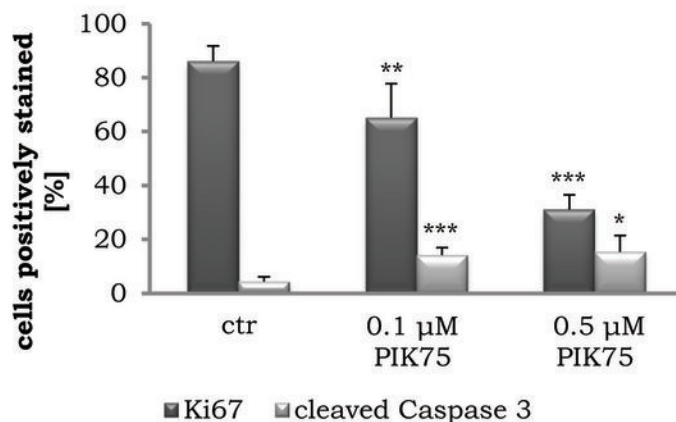
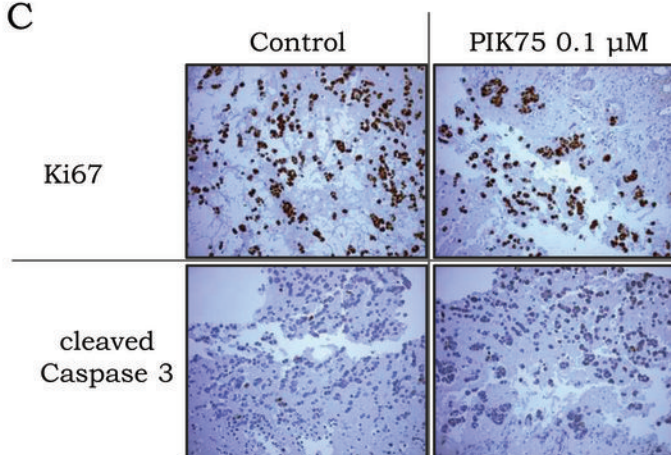


Figure 6

